Suppression of thermosensitive peptidyl-tRNA hydrolase mutation in *Escherichia coli* by gene duplication

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Peptidyl-tRNA hydrolase (Pth) in *Escherichia coli* is required to recycle tRNA molecules that dissociate from the ribosome as peptidyl-tRNA during protein synthesis. At non-permissive temperatures, strains with a thermosensitive mutation affecting the enzyme accumulate peptidyl-tRNA, cease protein synthesis and die. The rate of reversion of this mutation to thermoresistance varies widely according to the genetic background of the cell and the temperature of selection; under certain conditions, reversion can occur at rates approaching 10^{-3} per cell per generation. In such revertants, a chromosomal *pth* gene can be replaced by an inactivated gene, restoring thermosensitive growth in most cases. PCR amplification experiments and Southern blots show the presence of both normal and inactivated copies of the gene, demonstrating that gene duplication has occurred in the revertants. Estimation of intracellular peptidyl-tRNA hydrolase by Western blotting confirms this explanation of the mechanism of high-frequency reversion to thermoresistance.

Keywords: translation, ribosome, termination

INTRODUCTION

During normal protein synthesis, the ribosome forms initiation complexes on mRNA, performs repeated cycles of elongation as the mRNA is decoded, and releases the polypeptide chain by hydrolysis of peptidyl-tRNA when it encounters a termination signal on mRNA. However, protein synthesis is not perfectly processive, and peptidyl-tRNA dissociates prematurely from the ribosome at a frequency thought to be about 3×10^{-4} per codon (Jørgensen & Kurland, 1990; Manley, 1978). Peptidyl-tRNA released in the cell is hydrolysed by peptidyl-tRNA hydrolase (Pth), an enzyme that appears to be ubiquitous and has been shown to be essential in *Escherichia coli*. Pth is an esterase, able to recycle *N*-acetyl-aminoacyl-tRNA and peptidyl-tRNA by cleaving the ester bond between tRNA and peptide (Vogel *et al.*, 1968). In the absence of Pth activity, accumulation of some peptidyl-tRNA species in the cell is rapid, and protein synthesis becomes inhibited due to lack of aminoacyl-tRNA, leading to cell death. Certain tRNAs accumulate as peptidyl-tRNA much faster than others, and starvation in *E. coli* has been found to occur first for tRNA^{Lys} (Heurgué-Hamard *et al.*, 1996; Menninger, 1978).

Studies of premature peptidyl-tRNA dissociation (or

drop-off) have been greatly facilitated as a result of the isolation of a conditional lethal temperature-sensitive mutant affecting *pth* (Atherly & Menninger, 1972). Bacteria harbouring this mutation stop growing after a shift to non-permissive temperatures, following the accumulation of peptidyl-tRNA and the inhibition of protein synthesis. The mutation was identified as a single nucleotide change leading to an amino-acid substitution Gly100Asp (De La Vega *et al.*, 1996). Though distant from the active site proposed in the three-dimensional structure (Schmitt *et al.*, 1997), this Gly residue is highly conserved in Pth from many different organisms. Recent work has shown that the mutation leads to a Pth protein that is unstable *in vivo*, at both permissive and non-permissive temperatures, but which shows a specific activity comparable to that of the wild-type enzyme (Cruz-Vera *et al.*, 2000). The mutant enzyme seems not to be correctly folded and is subject to degradation by ClpP and Lon proteases (Cruz-Vera *et al.*, 2000).

In addition to allowing studies of peptidyl-tRNA accumulation, the thermosensitive Pth mutant has led to improved understanding of the drop-off process as a result of the isolation and characterization of extragenic revertants and multicopy suppressors of the *pth*(ts) mutation. Thus, overproduction of tRNA^{Lys} raises

considerably the threshold for thermosensitive growth by promoting more synthesis of the tRNA for which starvation occurs first (Heurgué-Hamard *et al.*, 1996). GroESL overproduction, presumably by improving the efficiency of mutant Pth protein folding, increases Pth activity. Apart from revertants directly affecting the *pth* gene, mutations inactivating termination factor RF3, or reducing the intracellular concentration of ribosome recycling factor RRF, suppress the *pth(ts)* mutation. *In vitro* experiments show that suppression is a result of a reduced frequency of peptidyl-tRNA drop-off in the cell (Heurgué-Hamard *et al.*, 1998). The studies that led to the isolation of these and other suppressors of *pth(ts)* showed that under some conditions of selection and in some strain backgrounds high rates of reversion to thermoresistance were observed. Here we examine this finding in more detail and show that the high rates of reversion are due to duplication of the *pth(ts)* gene.

METHODS

Bacterial strains and plasmids. *Escherichia coli* K-12 strains are listed in Table 1. FTP4993 and C600 were used as wild-type strains. JMM35 was constructed as follows. First, the kanamycin-resistance gene (Kn) from Tn903, cloned by flanking *Bam*HI sites (GenBank J01839, bp 697–2392) into the *Bam*HI site of the polylinker in BlueScribe M13+ (Stratagene), was excised by digestion with *Hinc*II and *Eco*RI and blunted by Klenow PolA. This fragment was cloned into the *Hinc*II site of the *pth(ts)* gene carried in plasmid pVH227 (a pWSK129 derivative, V. Heurgué-Hamard, UPR9073, CNRS, Paris, unpublished results), interrupting the Pth coding sequence after 28 amino acids. The *Kpn*I–*Pst*I fragment carrying the interrupted *pth* gene from this construction was then cloned into plasmid pMAK705, which is thermosensitive for replication (Hamilton *et al.*, 1989), giving plasmid pMAKpth::Kn. This plasmid allows recombination of *pth*::Kn at the *pth* chromosomal locus. Recombination and resolution of the integrated plasmid was performed as described by Blomfield *et al.* (1991) to obtain strain VH1004, which harbours *pth*::Kn in the chromosome of strain FTP4993 and a plasmid encoding wild-type *pth*, pMAKpth. A further plasmid carrying wild-type *pth* (pBADpth) was constructed by amplifying the *pth* gene with primers introducing *Eco*RI and *Hind*III sites at the beginning and end of the coding

sequence. These sites were then used to clone the fragment containing *pth* between the same sites in plasmid pBAD30 (Guzman *et al.*, 1995) cut by the same enzymes, placing the *pth* gene under the control of an arabinose-inducible promoter. Strain JMM35 was then constructed by P1 transduction of the *pth*::Kn locus from VH1004 to strain FTP4933 transformed with plasmid pBADpth in the presence of 0.2% arabinose. This strain depends on the presence of arabinose for normal growth.

Recombinant DNA techniques and genetic manipulations.

General procedures for recombinant DNA techniques, plasmid extraction, agarose gel electrophoresis, etc., were performed as described by Sambrook *et al.* (1989). DNA fragments from agarose gels were extracted using Jetsorb gel (Bioprobe) according to the manufacturer's instructions. Phage P1 lysates, transductions and transformations were performed as described by Miller (1992).

Growth conditions. Luria–Bertani broth (LB) was employed as rich medium. Antibiotics were added as necessary at the following final concentrations: 50 µg kanamycin ml⁻¹, 200 µg ampicillin ml⁻¹. Growth experiments at different temperatures were performed on agar plates. For pBAD30-derived plasmids, arabinose was added for induction at 0.02% for expression of *pth* at about chromosomal level or at 0.2% for maximum promoter activity.

Determination of reversion rates. Mutation rates were measured as described by Luria & Delbrück (1943). Serial dilutions (typically from 10⁻⁷ to 10⁻¹²) of an overnight culture of the *pth(ts)* mutant strains were made and grown overnight at 30 °C. The last dilution that grew was used to prepare 30 independent cultures each containing 20–50 bacterial cells in 200 µl rich medium. These were grown separately at permissive temperature (30 °C) for several hours, the period being determined by preliminary experiments such that about half the cultures acquired no revertants. Each culture was then completely plated on preheated agar plates and grown at non-permissive temperature. The number of plates on which no clones appeared allows determination of the mutation rate according to the equation $P = -(1/N)\ln F$, where P is the mutation rate per cell per generation, N the total number of bacterial cells per culture, and F the fraction of cultures acquiring no revertants (Luria & Delbrück, 1943).

PCR amplification of *pth* and *pth*::Kn. The presence of the intact and interrupted *pth* gene was verified by PCR using the pairs of oligonucleotide primers pt1-pt5, pt1-mk6 and mk2-

Table 1. Bacterial strains

Strain	Genotype	Derivation/use	Source
Xac	$\Delta(lac-pro)$ <i>argE ara gyrA rpoB thi</i>	<i>pth</i> wild-type	Coulondre & Miller (1977)
FTP4993	<i>metB glyV55 trpA</i> (AGC233, UGA234)	<i>pth</i> wild-type	F. T. Pagel, University of Texas
VH733	<i>metB glyV55 pth(ts)</i>	FTP4993 by several transductions	Heurgué-Hamard <i>et al.</i> (1998)
C600	<i>thr-1 leu-6 thi-1 supE44 tonA lacY1</i>	<i>pth</i> wild-type	G. Guarneros, CINVESTAN-IPN, Mexico City
VH4	C600, <i>pth(ts)</i>	C600 derivative	Heurgué-Hamard <i>et al.</i> (1996)
VH805	<i>metB glyV55 pth(ts) recA::Cm</i>	VH733 by transduction of <i>recA::Cm</i>	Menez <i>et al.</i> (2000)
VH1004	FTP4993, <i>pth</i> ::Kn, pMAKpth		This work
JMM35	FTP4993, <i>pth</i> ::Kn, pBADpth		This work
JMM101	FTP4993, <i>pth</i> ::Kn, pBADpth(ts)		This work

pt5 shown in Fig. 1. Primer sequences were: mk2, TGAT-GTTACAGATGAGATGGTC; mk6, CGCCTGAGCGAG-ACGA; pt1, GAATTC AATGGCACCGACGAAAATAC; pt5, GGGACTAACAGGCGGACA.

Western blotting. Western blot experiments were performed using rabbit anti-Pth antibodies commercially prepared from pure Pth protein. Cultures were centrifuged and the cells were lysed for 10 min at 100 °C in lysis buffer (50 mM Tris/HCl, pH 6.8, 100 mM DTT, 2% SDS, 0.1% bromophenol blue). Proteins were separated by electrophoresis on 15% polyacrylamide gels as described by Laemmli (1970). Gels were dried and the radioactivity in individual bands was determined when required using a phosphorimager (Molecular Dynamics). Transfer to nitrocellulose membranes and Western blotting with antibodies (diluted 5000-fold) were performed as described by Sambrook *et al.* (1989), using ¹²⁵I-labelled protein A (Amersham) to reveal the antibody.

Southern blotting. Following restriction enzyme digestion, DNA fragments were separated by gel electrophoresis in 0.7% agarose gels. Phage λ DNA digested with *Bst*II and labelled with [γ -³²P]ATP was used as molecular mass marker. A labelled probe for the *pth* gene was prepared by PCR amplification of the complete gene followed by labelling with a Ready Prime II labelling kit (Pharmacia) according to the manufacturer's instructions.

RESULTS

Reversion rate of the thermosensitive *pth* mutation depends strongly on selection temperature and genetic background

Previous studies have shown that the apparent frequency of reversion of the *pth(ts)* mutation isolated by Atherly & Menninger (1972) varies widely according to the non-permissive temperature employed, the plating medium and the strain background (Heurgué-Hamard *et al.*, 1996; V. Heurgué-Hamard & L. Mora, unpublished observations). Extragenic suppressor mutations such as the tRNA missense suppressor affecting the *glyW* gene were selected at temperatures of 8–10 °C above the threshold for thermosensitive growth, in strains with low apparent rates of reversion, consistent with point mutation rates (Heurgué-Hamard *et al.*, 1996). To obtain more quantitative data on mutation rates under different conditions, we determined the proportion of small independent cultures that acquired no revertants over a period of growth, as described by Luria & Delbrück (1943). These measurements allowed calculation of the reversion probability per cell per generation. Two *pth(ts)* strains were studied, VH733 and

VH4, with very different threshold temperatures for thermosensitivity (Table 2). In both cases, however, when the reversion rate was measured at 4 °C above this threshold, high rates of reversion were observed, in the range 10⁻³–10⁻⁵. The more thermosensitive strain, VH4, allowed the study of reversion rates over a range of temperature, and showed a striking decrease in reversion rate to 10⁻⁸ at 42 °C (Table 2).

Several factors may contribute to the differences, due to strain background and the temperature dependence of the reversion rate. The frequency of drop-off may vary between strains and is probably itself a temperature-dependent event (Cruz-Vera *et al.*, 2000). Residual Pth activity may be present in the strains at temperatures that are non-permissive for growth, and the level of such activity in relation to the volume of peptidyl-tRNA drop-off may determine the type of mutational event that restores growth. The level of Pth synthesis or the stability of thermosensitive Pth may vary between strains. Previous work had thrown light on the type of infrequent mutation that can suppress growth thermosensitivity of the mutant *pth* strain, but the nature of the reversion events observed at a high rate under certain conditions remained unknown. To distinguish between some of the possibilities outlined above we attempted to answer the following question: do the revertants arising at a high rate require residual Pth activity for growth?

Revertants selected under conditions of high reversion rate are due to duplication of the *pth* gene

A collection of *pth(ts)* revertants was made from a large number of independent cultures of the *pth(ts)* strain VH733 under conditions of high reversion rate, conserving one revertant only per culture. To determine whether the *pth* gene was still necessary for the growth of these revertants, a strain (JMM35) was constructed in which the chromosomal *pth* locus is interrupted by a cassette conferring kanamycin resistance, with the requirement for Pth met by a plasmid (pBADPth) carrying a wild-type *pth* gene expressed from an arabinose-inducible promoter. Each of the thermoresistant revertant strains, either before or after transformation with pBADPth, was transduced with a P1 lysate made on JMM35, selecting for kanamycin resistance in the presence of arabinose. Transductants of the thermosensitive control strain VH733 could only be obtained if the strain carried the plasmid pBADPth prior to transduction, as expected in view of the essential

Table 2. Rates of reversion to thermoresistance of *pth(ts)* strains

Strain	Threshold for thermosensitivity (°C)	Temp. of reversion (°C)	Reversion rate per cell per generation
VH733	39	43	5 × 10 ⁻⁴
VH4	34	42	10 ⁻⁸
VH4	34	40	2 × 10 ⁻⁶
VH4	34	38	10 ⁻⁵

Fig. 1. PCR analysis of DNA from thermoresistant revertants of strain VH733 after transduction of *pth::Kn*. DNA from thermoresistant revertants of the *pth(ts)* strain VH733 obtained at 43 °C, transduced to kanamycin resistance with *pth::Kn*, was analysed with PCR primers having sequences present in the parent strain (*pt1* and *pt5*) and in the kanamycin-resistant insert (*mk2* and *mk6*). Part (a) shows the positions of the primer pairs and the lengths of the expected PCR products. Part (b) shows the products obtained from amplifications on control strains VH733 [*pth(ts)*] and JMM35 (*pth::Kn*, pBAD), with a molecular mass ladder (100 pb); parts (c) and (d) show the products of amplification from six of the thermoresistant revertants after transduction to kanamycin resistance. Lanes are labelled with the numeric part of the primer pairs (thus, 1–6 is the pair *pt1*–*mk6*).

nature of the *pth* gene. In contrast, each of the thermoresistant revertant strains yielded about 200 kanamycin-resistant transductants whether or not the plasmid was present. Each of the plasmid-free trans-

duced revertants became once more thermosensitive, except in one case (numbered rev5). Similar experiments were performed with the more thermosensitive strain VH4, selecting thermoresistant revertants at 38 °C. In

this case also the revertants, but not the parent strain, could be transduced to kanamycin resistance with *pth::*Kn.

These observations suggested that thermoresistance had been acquired by duplication of the mutant *pth* gene. Three types of experiment were performed to test this hypothesis, by PCR amplification of the *pth* gene, Southern blot analysis, and Western blot analysis using anti-Pth sera. First, PCR amplifications were performed on revertants and transduced revertants. A pair of primers was chosen in the region neighbouring *pth* (see Fig. 1a), one upstream of the *pth* gene (pt1) and one within the coding sequence (pt5). In the case of both the transduced and non-transduced revertants, a fragment of the size corresponding to the normal *pth* gene was amplified, confirming the existence of a non-interrupted locus (Fig. 1b–d). A further specific primer (mk6) was chosen in the middle of the kanamycin-resistance gene introduced into *pth* which, in combination with pt1, amplified a fragment of the expected length (1.6 kb) only in the case of the transduced revertants, confirming the presence of the interrupted *pth* gene (Fig. 1b–d). Thus, it is clear that the revertants harbour at least two *pth* loci, one of which is interrupted on transduction with a P1 phage lysate on strain JMM35. The transduced revertants thus showed both the presence of a normal *pth* gene and a further interrupted copy of the gene (Fig. 1b).

Southern blot analysis was performed with probes specific to *pth* and to the kanamycin-resistance cassette on DNA extracted from FTP4993 and the 12 thermoresistant revertants after digestion with *EcoRV*. A single fragment was seen in each case (see Fig. 2b, c), of the expected size, 6.6 kb, as in the case of the VH733 parent strain (Fig. 2b, lane 2) and the Pth wild-type strain Xac (Fig. 2c, lane 2). After the transduction of the revertants with *pth::*Kn described above, a second fragment appeared in addition to that of 6.6 kb, of the size expected (11.6 kb) in the case of insertion of the kanamycin-resistance cassette into *pth*. Analysis of DNA from JMM35 showed no 6.6 kb fragment, but only the chromosomal inactivated fragment and a 4.8 kb molecule from the linearized pBADPth plasmid (Fig. 2b, lane 3). The identity of the more slowly migrating fragments was confirmed by hybridization first with the anti-Kn probe, followed by partial dehybridization and subsequent hybridization with the anti-*pth* probe (Fig. 2b, c). No new fragments were visible in the case of the revertants, suggesting that the region of the chromosome containing *pth* and undergoing duplication is probably larger than the region of about 6 kb covered by the restriction fragments.

Western blot analysis of Pth from different strains and thermoresistant revertants

The preceding experiments indicated that Pth is synthesized in the thermoresistant revertants even after inactivation of one chromosomal copy of the *pth* gene. To confirm this and look for possible differences in Pth

level in the revertants compared to the parent strain, we performed Western blot analysis on cell extracts. The level of Pth in strain VH733 was around the limit of detection in these assays, whereas all revertants showed clearly detectable Pth (Fig. 3). Inactivation of a chromosomal *pth* copy in the revertants by transduction of *pth::*Kn reduced the apparent level of Pth to near or below the level of detection except in the case of one revertant (rev5), the single case in which transduction did not restore thermosensitive growth to the revertant. Revertant rev5 may harbour more than two copies of the *pth* locus, by triplication for example. It is also possible that a duplication has placed the *pth* gene in a more expressed region, such as near the origin of replication.

The Western blot in Fig. 3 shows the slight difference in migration between wild-type Pth from strain FTP4993 and Pth from strains carrying the *pth(ts)* allele (Cruz-Vera *et al.*, 2000). Other experiments showed that the level of wild-type Pth in C600 and FTP4993 strains was closely similar, but insufficient sensitivity of the Western blotting technique precluded any comparison of Pth levels in the *pth(ts)* derivatives of these strains. Purified Pth enabled the Western blots to be calibrated, showing that both wild-type strains contain about 1000 molecules of Pth per cell (not shown). This is comparable to the value of 1300 molecules per cell found recently by Cruz-Vera *et al.* (2000) and significantly greater than an earlier report of 25 molecules per cell (Dutka *et al.*, 1993).

Reversion rate following shut-off of Pth synthesis

In the *pth(ts)* mutant the capacity of Pth to hydrolyse the peptidyl-tRNA dissociating from the ribosome diminishes with increasing temperature, until a threshold temperature is reached at which Pth activity becomes inadequate to sustain cell growth. This may be due to a reduction in Pth activity with temperature or to an increase in the rate of formation of cytoplasmic peptidyl-tRNA, the observations of Cruz-Vera *et al.* (2000) suggesting that the latter is of greater importance. In either case, the experiments described above show that the cell is able to counter an inadequate level of Pth activity within a certain range of temperatures above the threshold temperature for growth by multiplication of the number of copies of the mutant *pth* gene. This interpretation predicts that if the intracellular level of Pth is reduced to a small fraction of its value in the parent cell, this mechanism will no longer function. We therefore constructed strains in which Pth synthesis is under the control of a tight inducible promoter, and measured reversion frequencies after shut-off of *pth* expression.

Preliminary experiments with strain JMM35, in which wild-type Pth is expressed exclusively from the arabinose-inducible pBAD promoter on a low-copy-number plasmid, showed that many hours of growth were necessary after arabinose elimination to dilute the intracellular Pth. A similar result was obtained with the arabinose-inducible *pth* system recombined with the

Fig. 2. Southern blot analysis of DNA from thermoresistant revertants of strain VH733 before and after transduction of *pth::Kn*. Part (a) shows the *EcoRV* sites close to the *pth* gene and the expected size of the fragments containing the gene, before insertion of the *Kn* cassette. DNA from *pth* wild-type strain Xac, *pth*(ts) strain VH733, strain JMM35 in which the *pth* gene is carried on plasmid pBADp_{th}, seven thermoresistant revertants (rev3, 5, 6, 8, 9, 10 and 12) and their *pth*-interrupted derivatives (rev3 Kn, etc) were digested with *EcoRV* and subjected to Southern blot analysis. Two ³²P-labelled probes were used, specific to the *pth* gene (amplified between pt1 and pt5; see Fig. 1) and to the *Kn* cassette used to interrupt the *pth* locus (mk2 to mk6; Fig. 1). In each case, the two different probes were used successively on the same membrane, partially dehybridized after the first hybridization. Part (b) shows the results of hybridization with DNA from strains VH733, JMM35 and three thermoresistant revertants first hybridized with the *pth* probe and secondly with the *Kn* probe. Part (c) shows a similar analysis of five revertant strains, except that the anti-*Kn* probe was used before the anti-*pth* probe. The left track in (b) and (c) indicates marker fragments from phage λ DNA digested with *Bst*EII.

Fig. 3. Western blot analysis of proteins extracted from thermoresistant revertants of a *pth(ts)* mutant before and after introduction of *pth::Kn* by transduction. Western blotting with rabbit anti-Pth antibody was performed as described in Methods. Lanes rev2 Kn, rev3 Kn, etc., refer to strains after introduction of *pth::Kn* by transduction from strain JMM35. The other six revertants characterized were similar to the class including rev2, 3, 6, 9 and 11. The wild-type Pth strain FTP4993 and thermosensitive strain VH733 are included as controls.

chromosome at the maltose operon locus (data not shown). A plasmid was therefore constructed equivalent to pBAD_{pth} but encoding the thermosensitive enzyme, and used to transform FTP4993, in which the chromosomal *pth* gene was then inactivated as in JMM35. In the presence of 0.02% arabinose, the resulting strain is similar in behaviour to a 'chromosomal' *pth(ts)* strain. This construction has the advantage that upon elimination of arabinose not only is the synthesis of new Pth molecules arrested, but the short half-life of the thermosensitive Pth mutant results in a rapid decrease in the intracellular concentration of the enzyme. The reversion rate at 43 °C was studied as described above, and there was a total absence of revertants. We estimate that this corresponds to a reversion rate lower than 3×10^{-9} .

DISCUSSION

In certain genetic backgrounds and within a certain range of temperatures that are non-permissive for growth, the thermosensitive *pth* mutation reverts to thermoresistance at a high frequency. We have shown here that the mechanism of reversion is one of amplification of the mutant *pth* gene. With the exception of one of the revertants studied, the data are consistent with a simple duplication of the gene. This implies that a substantial range of conditions exists in which a modest increase in Pth activity is sufficient to restore cell growth.

At first sight, it might seem surprising that there should be such a critical level of Pth activity required for growth, such that a mere doubling in level of the enzyme at a given temperature should suffice to restore normal or near normal growth. A likely explanation for this is suggested by a consideration of the mechanism of peptidyl-tRNA drop-off from the ribosome. It was shown by Menninger (1978) that different families of peptidyl-tRNAs accumulate at rates differing by as

much as 200-fold after shift of a thermosensitive Pth strain to non-permissive temperature. Studies of the kinetics of drop-off of different peptidyl-tRNAs from the cognate codon on the ribosome have failed to show large differences in rate (Dinçbas *et al.*, 1999; Dinçbas-Renqvist *et al.*, 2000; V. Heurgué-Hamard, unpublished data). Likewise, the specificity of Pth towards different peptidyl-tRNA substrates does not show variations sufficient to account for the range of rates of peptidyl-tRNA accumulation (Dinçbas *et al.*, 1999; Dinçbas-Renqvist *et al.*, 2000; V. Heurgué-Hamard, unpublished data). These observations support the conclusion of Menninger (1978) that a substantial part of peptidyl-tRNA drop-off arises from translational errors; thus, drop-off occurs as a result of a weak semi-cognate codon-anticodon interaction rather than a cognate interaction. In this case, it is readily understandable that conditions under which Pth is rather limiting in the cell, leading to partial starvation for some species of aminoacyl-tRNA, will favour translational errors and increase yet further peptidyl-tRNA drop-off, imposing a greater substrate load on the available Pth. This may suffice to explain the need for a critical level of the enzyme.

Gene amplification is an extensively documented phenomenon in prokaryotic cells and can extend from simple duplication of a gene to amplification of more than 100-fold (for recent reviews, see Bachellier *et al.*, 1966; Hughes, 1999). Several examples in *E. coli* and *Salmonella typhimurium* have been described, such as the duplication at rates of 10^{-5} or more per cell per generation of a mutant Gly-tRNA synthetase gene (Folk & Berg, 1971), amplification of the *argF* region in some Hfr strains of *E. coli* (Jessop & Clugston, 1985) and amplification of the *lac* region (Tlsty *et al.*, 1984). Amplification is generally dependent on repeated sequences within the genome, such as the *rrn* genes (Anderson & Roth, 1981), insertion elements (Jessop &

Clugston, 1985), REP sequences (Shyamala *et al.*, 1990) or *rhs* repeated sequence elements (Lin *et al.*, 1984).

Although this has not been conclusively shown, it is probable that all currently identified mechanisms of suppression of the thermoresistance of *pth(ts)* depend on continued Pth activity in the cell. It is also clear that with the exception of the duplication mechanism shown here, reversion occurs at low frequency, of the order of 10^{-8} per cell per generation or lower, consistent with point-mutation events (Drake, 1969).

Saccharomyces cerevisiae chromosome VIII (Ouzounis *et al.*, 1995) and fragments of mammalian and mouse origin available in public databases reveal the existence of *pth* genes of the prokaryotic type. Biochemical studies of rabbit reticulocytes, however, have revealed another type of Pth activity, due to a phosphodiesterase which removes the terminal adenosine of tRNA together with the peptide, which appear then to be cleaved in a subsequent step (Gross *et al.*, 1992a, b). Recycling of the tRNA then requires repair of the 3' terminus by tRNA-CCA nucleotidyl transferase activity. It remains to be seen whether either the prokaryotic-type Pth activity or the phosphodiesterase-type Pth activity is essential to cell viability in cells of higher organisms.

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